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Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) or Virazole is a broad-spectrum antiviral agent whose molecular mode of action remains remarkably controversial. The drug was approved by the Food and Drug Administration in 1986 for aerosol use in infants with serious infections due to respiratory syncytial virus (RS) Ribavirin is and has been under clinical investigation against a variety of viral illness, including those due to influenza virus, Lassa fever, Korean hemorrhagic fever with renal syndrome (KHFS) and human immunodeficiency virus (HIV). There has been a great deal of clinical interest in utilizing ribavirn for HIV infections. It has been reported to slow the development of AIDS in HIV infected patients (1). We described here the major mechanisms of actions of this newly licensed drug.

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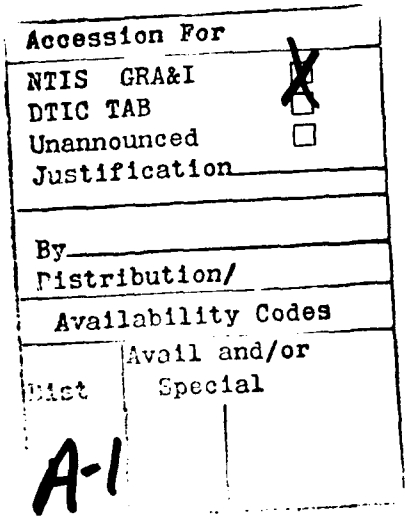

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INTRODUCTION

Ribavirin (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) or Virazole is a broad-spectrum antiviral agent whose molecular mode of action remains remarkably controversial. The drug was approved by the Food and Drug Administration in 1986 for aerosol use in infants with serious infections due to respiratory syncytial virus (RS). Ribavirin is and has been under clinical investigation against a variety of viral illness, including those due to influenza virus, Lassa fever, Korean hemorrhagic fever with renal syndrome (KHFS) and human immunodeficiency virus (HIV). The drug possesses inhibitory activity against a broad spectrum of viral pathogens, including both DNA and RNA viruses (2), intrinsically suggesting a vast clinical potential that has yet to be realized.

There has been a great deal of clinical interest in utilizing ribavirin for HIV infections. It has been reported to slow the development of AIDS in HIV infected patients (1).

Several theories regarding the molecular mode of action of ribavirin have been proposed. One hypothesis states that the drug leads to decreased intracellular pools of GTP, indirectly suppressing viral nucleic acid synthesis (4,5). Another hypothesis proposes that ribavirin therapy of viral-infected cells results in the synthesis of RNA with abnormal or absent 5' cap structures, which in turn leads to inefficient translation of viral transcripts (6). A third hypothesis states that the drug has a direct suppressive effect on viral polymerase activities. Experimentally it has been difficult to determine the primary mechanism of action because none of these hypotheses are mutually exclusive, and indeed, they may indicate that ribavirin acts in a complex, multiple site fashion. It is also important to note that ribavirin, unlike all other drugs whose structures resemble nucleoside analogues, has a modified

base. Antiviral agents, notably AZT, have modified sugars and generally are terminators of growing nucleic acid chains. Their major mode of action was relatively simple to determine.

Ribavirin Depletes Nucleotide Pools

Ribavirin was initially described as closely resembling guanosine in structure (7) and it was found that when cells were treated with this drug pools of GTP were diminished (8). Early after the development of this drug, ribavirin-5'-monophosphate was shown to be a potent inhibitor of IMP dehydrogenase activity (4). The hypothesis was made that the remaining cellular and viral enzymes were thought to compete for the depleted concentration of GTP. The major evidence against this being the primary mode of antiviral action is that other compounds notably, the 1,4,5-triazole derivative of ribavirin which deplete GTP pools, have no antiviral activity (8). Again, it is important to note that no mechanism excludes another and all could function, thereby enhancing the effect of the drug. Certainly if ribavirin does deplete nucleotide pools when it is also competing for these pools with viral enzymes, this mechanism, though not the main mechanism of inhibition, will clearly play an important role in antiviral activity.

Ribavirin therapy results in synthesis of RNA with abnormal or absent 5' cap structures.

The hypothesis that ribavirin exerts its antiviral effect by blocking capping of mRNA comes from early work with vaccinia virus. Katz et al (9) reported that ribavirin inhibited vaccinia virus growth in BSC I cells. Fifty ug/ml of virazole inhibited virus growth more than ten-fold, the effect, however, was not linear,

because 25 ug/ml caused only a two-fold decrease. Equimolar concentrations of guanosine added to cells resulted in loss of the inhibitory effect. In an assay measuring the appearance of viral particles ribavirin interfered dramatically. Their final conclusion was that ribavirin interfered with DNA synthesis in some unknown fashion. More recently Goswami et al (6) have used purified vaccinia virus capping enzymes and shown that ribavirin 5'-triphosphate was an effective inhibitor of the viral mRNA (guanine-7) methyltransferase, indeed by their kinetic analysis it appeared to be a direct competitive inhibitor. Canonico et al (10) reported that when examining mRNA from Eastern equine encephalitis virus infected cells that the transcripts appeared to have lost their cap structure.

In another reported set of experiments (12) in which the effects of ribavirin on vesicular stomatitis virus (VSV) grown in Chinese hamster ovary cells were examined, it was indicated that the drug leads to the synthesis of inefficiently translated viral mRNA. There was only a little demonstrable effect on viral primary transcription, lending support to the hypothesis that the drug alters RNA cap structures. Rankin et al (13) found that the reovirus mRNA synthesized in the presence of ribavirin triphosphate (RTP) could be translated as effciently as control mRNA. Also, ribavirin was not found to be incorporated into the RNA cap structure.

The problem with these and other experiments dealing with translational efficiencies and capping is that it is not clear that a cap is an absolute requirement for translation. Polio virus which does not have a cap on its mRNA translates quite adequately both <u>in vivo</u> and <u>in vitro</u>. Ironically, we have learned a great deal about the function, or lack thereof, of cap structures from this virus (14). More importantly, <u>in vitro</u> translation of mRNAs is notoriously unfaithful to the situation inside the infected cell. Rabbit reticulocyte lysate, in particular, will initiate internally on a message producing truncated proteins where none exist inside the

cell. Unfortunately, the assay to accurately measure translational efficiency is yet to be developed.

Our major goal was to understand the mechanism of action of ribavirin in two seperate systems, Vesicular stomatitis virus (VSV) and La Crosse virus (LAC). VSV is the prototype of a single segment negative stranded RNA virus and relatively insensitive to ribavirin. LAC virus is the prototype of a multiple segmented negative stranded RNA virus and is relatively senstitive to ribavirin. We believe we have fulfilled these major goals. We have also attempted to understand the mechanism of human immunodeficiency virus interference by ribavirin an important and unexpected outgrowth of our work, both for the Army and the scientific community in general.

BODY

Mechanism for the Bunyaviridae's extreme sensitivity to ribavirin.

Because of our inability to understand why bunyaviruses appear so exquisitely sensitive to this drug (15), we examined the effects of ribavirin on LaCrosse virus (LAC) virus infection (16), an important human pathogen. LAC virus is the prototypical bunyavirus and at the molecular level, certainly the best characterized. If indeed ribavirin proves to exert its primary antiviral effect by blocking cap formation of nascent viral mRNAs, then the observed sensitivity of bunyaviruses to this drug becomes quite puzzling, since the large numbers of stable, precapped cellular messages used to prime the mRNA are made prior to exposure to ribavirin and therefore should render the drug ineffectual. LAC is a member of the California encephalitis serogroup of Bunyaviruses (17) and an important pediatric pathogen.

The genome of the LAC virus consists of three segments of negative strand RNA, labeled small (S), medium (M), or large (L), each contained within a separate nucleocapsid (18). These nucleocapsids serve as templates for mRNA synthesis, initiating transcription by a cap-snatching mechanism similar to that of influenza virus (19,20). Like other negative strand viruses, LAC virus replicates through the synthesis of a full length plus-stranded intermediate, the antigenome.

A plaque reduction assay was used to initially measure the antiviral activity of ribavirin against LAC virus. Cell cultures overlayed with media containing 30 or 50 ug per ml of ribavirin showed complete inhibition of plaque formation. LAC is therefore relatively sensitive to inhibition by this drug. Our data supported the findings of Sidwell et al., (21), that similar concentrations of ribavirin are effective in inhibiting two strains of Punta Toro virus, another bunyavirus.

We attempted to determine whether the block in replication of the virus was at the level of mRNA transcription. S-mRNA synthesis was monitored as an indicator of transcriptional efficiency. Patterson et al.(20), reported that La Crosse virions contain an RNA-dependent RNA-polymerase which cleaves the host cell mRNA, 11 to 15 nucleotides from the 5' methylated cap, and uses this cleavage product as a primer during transcription of viral mRNAs. In order to first determine if this priming event was inhibited upon addition of ribavirin, total cellular mRNA and nucleocapsid RNA was isolated from cytoplasmic extracts using CsCl gradient isolated fractions from LAC infected cells (22). The S-mRNA was evaluated by primer extension analysis as described previously (20,23) and dot blot analysis. Primer extension products represent extensions of mRNAs that have 50 bases of (-) genome encoded message plus an 11 to 15 base nontemplated 5' extension derived from precapped host cell mRNA (20). When the concentration of ribavirin added to the host cells during transcription is increased from 10 to 30 ug/ml there is a five unit drop in the relative area of the mRNA (20% of the total scale) as shown in Figure 1 (Cassidy and Patterson, 1989). No further decrease was observed when the concentration of ribavirin was raised from 30 to 50 ug/ml.

The effect of ribavirin does not seem to change the primer, since the length and appearance of the 5'cap remains the same (Fig. 1, Cassidy and Patterson, 1989). There is however, a significant quantitative decrease in the total amount of mRNA produced. Our results further made the point that the inhibition is the same at the 5' end as that seen further downstream suggesting the inhibition occurs at initiation of transcription. The inhibition of LAC virus production appears to occur early in the replicative cycle. Antiviral activity is observed within 6 hrs postinfection, during primary transcription of the mRNA.

Stable antigenome RNAs of LAC are exclusively found in nucleocapsid structures. To determine whether the presence of ribavirin would effect antigenome

synthesis, we isolated nucleocapsid structures from LAC infected cells that had been treated with ribavirin. When 30 to 50 ug/ml of ribavirin were added antigenome production was not detected in a dot blot analysis, although some mRNA can still be detected at these concentrations of ribavirin.

The overall scheme for the replication of LAC virus is similar to that used by other negative-strand RNA viruses, such as RSV, Lassa fever and VSV. Following primary transcription, nucleocapsid assembly takes place resulting in the production of a full length antigenome (22). Continued protein synthesis is a requirement for antigenome production. It might be expected that the inhibition of antigenome synthesis would be directly proportional to the inhibition of mRNA synthesis. However, this is not the case. The addition of ribavirin diminishes antigenome production more than it does mRNA synthesis. Although it cannot be ruled out that there is a direct block on antigenome RNA synthesis, more than likely, the reduction in the concentration of mRNA results in a lowered amount of viral proteins and subsequently a block in nucleocapsid assembly (24). Inhibition of viral progeny, in turn, is more sensitive to ribavirin then nucleocapsid inhibition. Our data suggests that ribavirin's major effect is on LAC viral RNA-dependent RNA-polymerase activity (16).

The marked susceptibility of LAC virus to ribavirin may be important in treating the intracerebral infections that result from LAC infection. Ferrara et al.,(25) reported that ribavirin inefficiently crossed the blood-brain barrier and was unable to accumulate in the brains of animals. However, subsequently two groups have been able to measure a maximum concentration of 9.5 uM of ribavirin in the spinal fluid of patients receiving high-dose oral therapy (26,27). Ribavirin may be an important therapeutic agent for treating human LAC virus and other California serotype infections, considering that the level of ribavirin required for inhibition, in tissue culture, of LAC virus (0.3 uM) is significantly lower than the concentration

found in the CSF. There are implications for other forms of encephalitis, particularly those associated with RNA viruses, but each infecting agent must be analyzed seperately.

Inhibition of Viral RNA-dependent RNA polymerases.

The idea that ribavirin may directly affect polymerases has been reported previously for other viruses, notably influenza virus (28). Work in our lab has indicated that the structural similarity of ribavirin may include all of the natural substrates of these enzymes, all four of the nucleoside triphosphates. We chose to examine ribavirin's effect on the VSV system because it is perhaps the best studied RNA virus. It is an extremely well defined system.

Results of our experiments in which the effects of phosphorylated ribavirin compounds on an in vitro VSV polymerase assay indicated that the drug does indeed possess a significant direct suppressive effect on viral polymerase (29). In fact all three phosphorylated species inhibited VSV transcription. The mono- and diphosphorylated forms of the drug possessed approximately two to three times the inhibitory activity as the triphosphorylated form. Transcripts synthesized in the presence of the drug were full-length. Inhibition by ribavirin 5'-diphosphate could be reversed by the addition of UTP, CTP and GTP. The addition of GDP to the reaction did not reverse inhibition as shown in Table 1 (Toltzis et al, 1988). This supports the notion that RDP was not being converted to RTP because no GDP could be converted to its triphosphorylated form and thereby reverse the reaction. Nearest neighbor analysis supported the notion that none of the phosphorylated forms were incorporated into the growing chain of RNA as shown in Figure 2 (Toltzis et al, 1988). An inability to incorporate into the RNA has also been reported with reovirus transcription (13).

Surprisingly, in contrast to the viral mRNA synthesized in infected cells in the presence of ribavirin, transcripts synthesized in vitro, i.e. in the test tube, in the presence 5' mono-, di-, and triphosphorylated forms of the drug, translated with equal efficiencies under the test conditions. This results either suggest a contradiction to the theory that ribavirin affects cap structures and interferes with translation, or that the in vitro RNA synthesis system cannot mimic the cellular machinery accurately.

Our initial observation that besides the triphosphorylated form both the mono- and di-phosphorylated forms of the drug were active against the VSV replicase was surprising. It has been shown previously that all nucleoside analogs inhibit viral polymerases in the triphosphorylated form (30,31).

We have used enzyme kinetic procedures and product analysis to further investigate the mechanism of action of ribavirin. When analyzed by double-reciprocal plots both RDP and RTP gave similar patterns of inhibition, although the Km values of the nucleoside triphosphates in the presence of the different drugs are not the same (32). This type of analysis is shown on Figure 3 (Fernandez-Larsson et al., 1989). Both of the phosphorylated forms of ribavirin compete directly with all four of the nucleoside triphosphates, suggesting that they are acting on the polymerase in a similar fashion. Because there appears to be no incorporation of ribavirin into the growing nucleic acid chain and because the transcripts synthesized appear full length in the presence of both drugs it was our initial hypothesis that RDP and RTP were blocking polymerase initiation at the 3' terminus of the genome. To confirm our results regarding the affects of ribavirin-diphosphate we examined three analogues which stabilize as diphosphates in our VSV in vitro transcription reaction. Although these compounds are not as inhibitory as RDP, they did inhibit the transcription reaction and support the hypothesis that RDP can act on its own to suppress the VSV replicase (Lachemann et al., 1990).

We then studied purified in vitro transcription products synthesized in the presence and absence of phosphorylated ribavirin compounds to determine the effect of these drugs on leader RNA and mRNA synthesis and to further characterize the observations on enzyme kinetics. VSV leader RNA is a 47 nucleotide noncapped and nonpolyadenylated RNA transcript which is the first RNA synthesized after the polymerase begins transcription, it is complimentary to the exact 3' end of the negative stranded RNA genome. The next transcript synthesized is the nucleocapsid (N) mRNA. It is both capped and polyadenylated. We found that the inhibition of leader synthesis was similar to the inhibition of N mRNA synthesis, since the ratios of these two RNA transcripts did not appear to change with the addition of RDP or RTP to the transcription reaction when the primer was in excess (32). These results are shown in Table 2. The results suggest that the inhibitory effect of ribavirin on the polymerase occurs at the level of initiation of primary transcription, when the transcriptase enters the 3' end of the genome. Later, using the same type of analysis we were able to show that a VSV mutant had an altered ATP function. Kinetic analysis clearly showed that the direct competitive effect of RDP or RTP was different in this mutant (33). The altered ATP function observed in the kinetic analysis is compatible with the argument for an additional ATP-binding site on the polymerase complex. In this mutant RTP and RDP seemed to interact in a different manner with the ATP binding sites than they do with wild-type VSV.

In the only other well studied RNA viral system looking at the mechanism of action of ribavirin is the reovirus system. Reovirus is a segmented dsRNA virus. Here, researchers reported that 12.5 uM of ribavirin inhibits all stages of viral replication (13). When RTP was tested on an in vitro transcription reaction utilizing the dsRNA genomes as template, both elongation and initiation of plus stranded mRNAs were inhibited. In this system elongation, rather than initiation of

transcription was more sensitive to RTP when looking at transcriptionally activity reovirus cores. As stated previously there was no effect on cap formation and/or methylation nor was ribavirin incorporated into the growing chain. These results are similar to the VSV situation where no effect on apparent in vitro translatebility could be found when mRNA was synthesized in vitro. These authors have suggested a model that supports RTP binding to a catalytic site on the transcriptase, again similarly to the VSV system.

Our data and others have suggested that ribavirin has an effect on transcription by RNA-dependent RNA polymerases and that this effect may be mediated by several phosphorylated forms. One of the reasons that the antiviral activity of ribavirin maybe so formidable to pin down is that the inhibition of polymerases, without specific chain termination, is more difficult to assess. However, if indeed primary transcription is affected either at initiation or elongation, direct linear effects of the drug on later stages in viral replication and assembly are difficult to ascertain as any deregulation of the system at the mRNA level is presumably amplified at all levels of viral progeny production. These small effects on primary transcription may be enough to produce the entire antiviral activity seen, with respect to diminished viral titres.

MECHANISM OF ACTION OF HIV INTERFERENCE WITH RIBAVIRIN

This work has recently been accepted for publication in MOLECULAR PHARMACOLOGY. A copy of the article is enclosed in this report. The work is described below.

First, studies were conducted with the reverse transcriptase of AMV in an *in vitro* system and it was found that RDP, RTP and ribavirin were inhibitory of the enzyme. These results prompted us to investigate the effect of the drugs on HIV-1

RT.

RTP caused a marked inhibition of cloned HIV RT when added at time zero to an *in vitro* assay with poly(A)$_n$-oligo(dT)$_{10}$ as primer-template. The inhibition was almost complete (98%) at a concentration of 309 uM (150 ug/ml), irrespective of the time at which the synthesis of DNA was measured. At a concentration of 206 uM (100 ug/ml) RTP affected a 79% inhibition of the RT reaction, and at 103 uM (50 ug/ml) 47% of the reaction was inhibited. From the values obtained for the reaction mixtures which had been incubated for 30 minutes a dose response curve was obtained from which the ID_{50} of RTP was estimated for the *in vitro* HIV RT reaction at about 112 uM (54 ug/ml).

RDP had a higher inhibition than RTP in an identical HIV RT *in vitro* assay. The inhibition was 100% at 371 uM (150 ug/ml). At 30 minutes of incubation, 198 uM (80 ug/ml) of RDP affected a 92% inhibition of the RT reaction, 99 uM (40 ug/ml) a 60% inhibition, and at 49 uM (20 ug/ml) RDP still caused a 31% inhibition. From the dose response curve the ID_{50} was estimated to be about 81 uM (33 ug/ml). RDP was almost 40% more inhibitory than RTP.

Unphosphorylated ribavirin was a much less effective inhibitor of HIV RT and AMV RT. At 30 minutes of incubation, 615 uM (150 ug/ml) of ribavirin caused a 42% inhibition of HIV RT. This percentage of inhibition could be attained with 128 uM RTP, as extrapolated from its dose response curve. Similarly, it was found that the same concentration of ribavirin (615 uM) caused a 58 % inhibition of AMV RT, while this same inhibition could be achived by 82 uM of the triphosphate.

Other viral polymerase inhibitors as AZT, which are known to be effective when triphosphorylated, do so by chain termination. A Sanger chain termination reaction with RTP was performed and found no chain termination in the presence

of this drug. This is in agreement with our previous finding with the VSV polymerase that transcripts synthesized in the presence of drug were full length and were absent of incorporated drug.

In order to determine the mechanism of inhibition we performed a kinetic analysis of the ability of RTP and RDP to inhibit the cloned HIV RT. The K_m for dTTP, without the inhibitor, was 57 nmoles $\pm$ 4. It was found that in several repetitions of the experiment this value was consistent, and significantly lower than K_m values obtained previously with purified HIV RT. The difference was in the order of 10 to 100-fold lower than published results, depending on the study. The K_m value is also lower than a previous report where a cloned HIV RT was used. The difference between these values could be attributed to the inherent characteristics of our particular cloned HIV RT, to the differences between affinity-purified native HIV RT and cloned HIV RT, or both.

Even though RTP was an inhibitor of HIV RT, the type of inhibition and the K_i value could not be obtained using standard Lineweaver-Burk plots. The double-reciprocal plot of the inhibition of HIV RT by RTP showed a non-linear sigmoid dependence of velocity upon substrate concentration, regardless of the different concentrations of RTP used (only one RTP concentration shown). The sigmoid behavior is also clearly seen in a replot of [S]/Vo against Vo.

Further more when dideoxyinosine (DDI) was tested in combination with any of the ribavirin derivatives a precisely additive inhibitory effect in the *in vitro* assay.

In summary: Ribavirin inhibits the human immunodeficiency virus (HIV) reverse transcriptase (RT) in an *in vitro* reaction. Ribavirin-5'-diphosphate was close to 40% more inhibitory than ribavirin-5'-triphosphate (RTP).

Unphosphorylated ribavirin had a reduced, but detectable, effect as an inhibitor than the phosphorylated forms. The compounds seem to have a direct effect on the viral polymerase, and no chain termination was observed in the presence of RTP. Combination of any of the ribavirin derivatives tested with 3'-azido-3'-deoxythymidine (AZT, zidovudine) 5'-triphosphate resulted in an increase of its anti-HIV RT activity in the *in vitro* assay. DDI and ribavirin also show an additive effect in vitro.

CONCLUSIONS

All antiviral compounds must be evaluated acknowledging, that by the time "self-limiting" diseases are recognized most replication and viral progeny production is accomplished. The damage is done, so to speak. It is not fair to compare these compounds with antibacteriocidal agents. The reality is that most of the effective antivirals will only work on chronic infections. Nevertheless, as the development of antiviral compounds grows, we need to adequately prepare ourselves to determine, primarily via rapid viral diagnostics, which virus is the agent of infection. The length of the viral infection is also critical information. If early events of replication are the sole target of ribavirin's antiviral activity then only early use of the drug would prove to be efficacious.

We plan to continue to evaluate the mechanisms of inhibition of ribavirin on RNA viruses, particularly the bunyaviruses. The Bunyaviridae include viruses which have been implicated in a wide variety of human diseases such as Rift Valley Fever, Congo-Crimean Hemorrhagic Fever and Hemorrhagic Fever with Renal Syndrome (HFRS) all of which cause world wide morbidity and mortality (34). Studies indicate that Hantaan-related viruses (etiologic agent of HFRS) infect rats in widespread areas of the US: in areas close to coasts as well as inland areas such as Columbus, Ohio (35). Recently, Hantaan virus has been found to infect human populations in Maryland (36). It is of particular interest to us that in vivo, ribavirin-treated mice are resistant to Hantaan virus infection and furthermore infant mice receiving one dose of ribavirin are protected against the fatal meningoencephalitis associated with Hantaan virus (37,38,39).

Our work with combinations of chain terminators and ribavirin on HIV in vitro suggest that combination therapy may have very beneficial effects on the clinical course of AIDS.

Ribavirin remains an important potential agent in the treatment of a broad spectrum of serious viral illnesses encountered worldwide and hopefully will serve as a model for similar, more effective agents in the future. Definition of its molecular activity will be vital to the development of its use and the use of similar agents.

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APPENDIX

RIBAVIRIN IS AN INHIBITOR OF HIV REVERSE TRANSCRIPTASE*

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SUMMARY

Ribavirin inhibits the human immunodeficiency virus (HIV) reverse transcriptase (RT) in an *in vitro* reaction. Ribavirin-5'-diphosphate was close to 40% more inhibitory than ribavirin-5'-triphosphate (RTP). Unphosphorylated ribavirin had a reduced, but detectable, effect as an inhibitor than the phosphorylated forms. The compounds seem to have a direct effect on the viral polymerase, and no chain termination was observed in the presence of RTP. Combination of any of the ribavirin derivatives tested with 3'-azido-3'-deoxythymidine (AZT, zidovudine) 5'-triphosphate resulted in an increase of its anti-HIV RT activity in the *in vitro* assay.

INTRODUCTION

Antiviral compounds have attracted considerable attention as potential therapeutic agents against AIDS[1] almost since the causative agent, now referred to as HIV, was first identified as a retrovirus. To date, interest has focused on selective antiviral compounds targeted against the viral RT, the CD4 cellular receptor molecule for the virus, and more recently, the protease encoded by HIV (1-4). The most potent inhibitors of HIV RT studied have been the nucleoside analogs. To this group belong the 2',3'-dideoxynucleosides which apparently excert their inhibition by chain termination of the growing DNA chain (5, 6). The best known example is AZT, which is the only drug approved for the treatment of HIV infection in the U.S.

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[1]**ABBREVIATIONS:** AIDS, acquired immunodeficiency syndrome; AMV, avian myeloblastosis virus; AZT, 3'-azido-3'-deoxythymidine; AZT-TP, AZT-5'-triphosphate; HIV, human immunodeficiency virus; ID_{50}, inhibitory dose 50%; RDP, ribavirin-5'-diphosphate; RT, reverse transcriptase; RTP, ribavirin-5'-triphosphate; VSV, vesicular stomatitis virus.

Ribavirin (1-beta-D-ribofuranosyl-1,2.3,-triazole-3-carboxamide) is a nucleoside analogue of purines with a broad spectrum antiviral activity against many RNA and DNA viruses (7, 8). It is licensed in the U.S. for the treatment of pediatric respiratory syncytial virus infection, but it has been used successfully in the treatment of other viral diseases (9). The long history of this drug, as well as its documented toxicity and safety evaluations has warranted the study of ribavirin against HIV. Ribavirin inhibits HIV-1 infection of cultures of human adult T lymphocytes (10). It has been reported to delay or prevent development of AIDS in patients with HIV-induced persistent generalized lymphadenopathy (11). The mechanism by which ribavirin might interfere with the replication of the virus has not been studied.

Several theories have been proposed to explain the molecular mode of action of ribavirin. One proposed mechanism states that the monophosphate derivative of ribavirin is a potent competitive inhibitor of IMP dehydrogenase, therefore disrupting the de novo synthesis of GMP and causing a depletion of the GTP pools in the cell (12, 13). However, a derivative of ribavirin which depletes GTP pools, has no antiviral activity (14). A second proposed mechanism suggests that ribavirin treatment of viral infected cells results in the synthesis of mRNA with absent or abnormal cap formation (15). This theory is supported by the observation that poliovirus, which lacks a cap at the genomic 5' end, is not significantly inhibited by ribavirin, and that ribavirin's effect can be reversed by the addition of guanosine (12, 13, 16).

The third hypothesis states that ribavirin has a direct suppressive effect on the viral polymerase (17-21). It must be noted that these hypotheses are not mutually exclusive and may be an indication that ribavirin acts in a multiple site fashion. We have previously reported that ribavirin has a significant direct effect on the viral polymerase in a VSV *in vitro* transcription system (20). This effect did not involve chain termination and was mediated to different degrees by RDP and RTP. Inhibition by RDP or RTP could be

reversed by addition of GTP, CTP and UTP, but not by the addition of GDP or ATP (20). Recently, when we examined the kinetics of the effect of phosphorylated ribavirin on the VSV *in vitro* transcription reaction we found that both RDP and RTP were competitive inhibitors with all four natural nucleoside triphosphates (21).

We report another inhibitory effect of ribavirin against a viral polymerase, the RNA-directed DNA polymerase associated with HIV-1, its probable mechanism of action, and its interaction with AZT.

MATERIALS AND METHODS

Chemicals. The phosphorylated ribavirin compounds and AZT-TP were kind gifts from Roland K. Robins, Nucleic Acid Research Institute, Costa Mesa, CA, and Phillip A. Furman, Burroughs Wellcome Co., Research Triangle Park, NC, respectively. Non-phosphorylated ribavirin (Virazole^{R}) was obtained from ICN Pharmaceuticals, Inc., Costa Mesa, CA.

Enzymes. AMV RT was a commercial product purified from virions, obtained from Promega Corporation. Cloned HIV-1 RT (clone BH-10) obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, National Institutes of Health, was used throughout this study.

RT in vitro reaction. Reaction mixtures (150 ul) contained 50 mM Tris HCl (pH 7.3), 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 ug poly(rA)-oligo$(dT)_{10}$, 0.133 mM alpha [^{32}P]dTTP (New England Nuclear), 0.5 U of cloned HIV RT, and the indicated amount of RTP or RDP (one unit of HIV RT was defined as the amount of enzyme which catalized the incorporation of one nmoles of TMP into DNA in 10 min. at 37^{o}C). For AMV RT experiments, the conditions of the reactions were identical as above, excepting the enzyme. Each tube contained 0.5 U of AMV RT (one unit of AMV RT was defined as the amount

of enzyme which catalized the incorporation of 1 nmoles of dTTP into acid-insoluble form in 10 minutes at 37^{o}C).

Dideoxy chain termination reaction. The Sanger (22) dideoxy chain termination sequencing procedure was used to determine if RTP could function as a chain terminator in a growing DNA chain (Fig. 2). The template/primer was a pGEM-2 double-stranded DNA plasmid and an 18-mer synthetic oligonucleotide complementary to the T7 promoter region of pGEM-2. The primer was labelled at its 5' end with [gamma-^{32}P]ATP and T4 polynucleotide kinase, and preannealed to plasmid template. The reaction mixtures (5 ul) contained 50 mM Tris HCl (pH 7.3); 100 mM KCl; 5 mM $MgCl_2$; 1 mM DTT; 200 uM each of dGTP, dATP, dTTP, and dCTP; .7ug pGEM-2DNA, 1 ug of oligonucleotide primer and 1.4 U of AMV reverse transcriptase. Each of the reactions in lanes G, A, T, C, and RTP, also contained 40 uM ddGTP, 40 uM ddATP, 40 uM ddTTP, 40 uM ddCTP, and 160 uM RTP, respectively. Lane Blank contained no dideoxy nucleotides or RTP. Incubation was for 20 minutes at 42^{o}C, at which time the reactions were stopped by adding 5 ul of a formamide loading buffer, heated for 2 minutes at 100^{o}C and analyzed on a sequencing gel (24). RTP did not cause any base-specific chain termination.

RESULTS

First, studies were conducted with the reverse transcriptase of AMV in an *in vitro* system and it was found that RDP, RTP and ribavirin were inhibitory of the enzyme (Table 1). These results prompted us to investigate the effect of the drugs on HIV-1 RT.

RTP caused a marked inhibition of cloned HIV RT when added at time zero to an *in vitro* assay with poly(A)$_n$-oligo(dT)$_{10}$ as primer-template (Fig. 1, A). The inhibition was almost complete (98%) at a concentration of 309 uM (150 ug/ml), irrespective of the time at which the synthesis of DNA was measured. At a concentration of 206 uM (100 ug/ml) RTP affected a 79% inhibition of the RT reaction, and at 103 uM (50 ug/ml) 47% of the

reaction was inhibited. From the values obtained for the reaction mixtures which had been incubated for 30 minutes a dose response curve was obtained (Fig. 1, A; insert) from which the ID_{50} of RTP was estimated for the *in vitro* HIV RT reaction at about 112 uM (54 ug/ml).

The effect of RDP was also studied as it was previously shown that this molecule was more inhibitory to viral RNA-dependent RNA polymerases than RTP (20, 21). RDP had a higher inhibition than RTP in an identical HIV RT *in vitro* assay (Fig. 1, B). The inhibition was 100% at 371 uM (150 ug/ml). At 30 minutes of incubation, 198 uM (80 ug/ml) of RDP affected a 92% inhibition of the RT reaction, 99 uM (40 ug/ml) a 60% inhibition, and at 49 uM (20 ug/ml) RDP still caused a 31% inhibition. From the dose response curve (Fig. 1, B; insert) the ID_{50} was estimated to be about 81 uM (33 ug/ml). RDP was almost 40% more inhibitory than RTP.

Unphosphorylated ribavirin was a much less effective inhibitor of HIV RT and AMV RT. At 30 minutes of incubation, 615 uM (150 ug/ml) of ribavirin caused a 42% inhibition of HIV RT. This percentage of inhibition could be attained with 128 uM RTP, as extrapolated from its dose response curve. Similarly, it was found that the same concentration of ribavirin (615 uM) caused a 58 % inhibition of AMV RT, while this same inhibition could be achived by 82 uM of the triphosphate.

Other viral polymerase inhibitors as AZT, which are known to be effective when triphosphorylated, do so by chain termination (25). A Sanger chain termination reaction

(22) with RTP was performed and found no chain termination in the presence of this drug (Fig. 2). This is in agreement with our previous finding with the VSV polymerase that transcripts synthesized in the presence of drug were full length and were absent of incorporated drug (20).

In order to determine the mechanism of inhibition we performed a kinetic analysis of the ability of RTP and RDP to inhibit the cloned HIV RT. The K_m for dTTP, without the inhibitor, was 57 nmoles $\pm$ 4. It was found that in several repetitions of the experiment this value was consistent, and significantly lower than K_m values obtained previously with purified HIV RT (26, 27, 25, 28, 29). The difference was in the order of 10 to 100-fold lower than published results, depending on the study. The K_m value is also lower than a previous report where a cloned HIV RT was used (30). The difference between these values could be attributed to the inherent characteristics of our particular cloned HIV RT, to the differences between affinity-purified native HIV RT and cloned HIV RT, or both.

Even though RTP was an inhibitor of HIV RT, the type of inhibition and the K_i value could not be obtained using standard Lineweaver-Burk plots. The double-reciprocal plot of the inhibition of HIV RT by RTP showed a non-linear sigmoid dependence of velocity upon substrate concentration (Fig. 3, left), regardless of the different concentrations of RTP used (only one RTP concentration shown). The sigmoid behavior is also clearly seen in a replot of [S]/Vo against Vo (Fig. 3, right).

DISCUSSION

As we have reported previously (for review see Patterson and Fernandez-Larsson, ms in press) ribavirin's structural similarity may include all of the natural substrates of some polymerases. Results of our experiments in which the effects of phosphorylated ribavirin compounds on an in vitro vesicular stomatitis virus polymerase assay indicated that the drug does indeed possess a significant direct suppressive effect on viral

polymerase. All three phosphorylated species inhibited VSV transcription. The mono-and diphosphorylated forms of the drug possessed approximately two to three times the inhibitory activity as the triphosphorylated form. Transcripts synthesized in the presence of the drug were full-length. Inhibition by ribavirin 5'-diphosphate could be reversed by the addition of UTP, CTP and GTP and the addition of GDP to the reaction did not reverse the inhibition. The observation that besides the triphosphorylated form both the mono- and diphosphorylated forms of the drug were active against the VSV replicase was surprising. We have used enzyme kinetic procedures and product analysis to further investigate the mechanism of action of ribavirin. When analyzed by double-reciprocal plots both RDP and RTP gave similar patterns of inhibition, although the Km values of the nucleoside triphosphates in the presence of the different drugs not identical. The Km of RDP reflected its greater inhibitory effect. Both of the phosphorylated forms of ribavirin compete directly with all four of the nucleoside triphosphates, suggesting that they are acting on the polymerase in a similar fashion. Because there appears to be no incorporation of ribavirin into the growing nueclic acid chain and because the transcripts synthesized appear full length in the presence of either drug it was our hypothesis that RDP and RTP were blocking polymerase initiation at the 3' terminus of the genome.

Ribavirin and its phosphorylated derivatives inhibit HIV RT in an in vitro reaction. The finding that RDP is a better inhibitor than RTP suggests that the modified base of ribavirin and possibly a phosphate charge are the significant factors in this non-chain terminating interference. The number of phosphates do not seem to be critical, although the presence of the charge might be the potentiating factor when there is no phosphodiester bond involvement. As mentioned already, nonphosphorylated ribavirin had a substantialy lower inhibitory effect on both HIV RT and AMV RT, when compared to the phosphorylated compounds.

We were unable to determine the type of inhibition caused by RTP on HIV RT

using standard plots. Within the scope of this paper, the results of this graphic analysis are interpreted as cooperativity or allostery (31). However, it is important to note that we have previously found that phosphorylated ribavirin compounds unmasked an allosteric site, in that case presumably an ATP-binding site, on the transcriptase of a mutant vesicular stomatitis virus (23). It is therefore possible that the abnormal kinetic behavior we see with HIV RT in the presence of RTP is also caused by this molecule acting on a site other than the nucleotide polymerization site, and it is likely that the anti-HIV activity of ribavirin is based on a direct effect on the viral polymerase.

In considering a combination of antiviral drugs that may lead to an increase in the efficacy of available treatments, ribavirin has been tested *in vitro* and found to enhance the effects of 2',3'-dideoxy purine analogues (32), but antagonize the inhibitory effects of 2',3'-dideoxy pyrimidine analogues. In the case of 3'-azido-2,6-diamino purine-2',3'-dideoxyriboside (AzddDAPR), combination of this drug with ribavirin also extended the enhancement of its inhibitory activity *in vivo* (33). We were interested to determine the effect of a combination of ribavirin and AZT on the *in vitro* reverse transcriptase reaction since an antagonism on HIV replication has been reported between both drugs (34). It was found that unphosphorylated ribavirin, RDP or RTP did not antagonize but rather increased the inhibition of the 5'-triphosphate of AZT on the HIV RT (Fig. 4). This result was not totally unexpected in view of the very different mechanisms of action of AZT and ribavirin. Also, in the *in vitro* reaction the drugs are stabilized, whereas in the infected cell both drugs undergo similar phosphorylation events that might cause them to antagonize each other. In a clinical situation, that undesirable effect could be avoided if at least one of the drugs is delivered as a stabilized phosphorylated compound. We have previously demonstrated that stabilized ribavirin 5'-diphosphate derivatives retain antiviral activity (35). These phosphorylated ribavirin compounds could not passively diffuse through the cell membrane, but could ostensibly be delivered in a liposome system (36). Our observation that RDP has the highest inhibitory activity of the ribavirin compounds is

significant in this context.

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Figure 1. Inhibition of HIV-1 RT by (A) ribavirin-5'-triphosphate and (B) ribavirin-5'-diphosphate. The reactions were incubated at 37^{o}C for 45 min. Samples (35 ul) of each of the reaction mixtures were removed at 0, 15, 30, and 45 min. for measurement of standard trichloroacetic acid-soluble radioactivity.

Figure 2. Dideoxy chain termination reactions using AMV RT. Lanes G, A, T, and C contained 40 uM ddGTP, 40 uM ddATP, 40 uM ddTTP, and 40 uM ddCTP, respectively. Lane RTP contained 160 uM RTP and lane B contained no dideoxy nucleotides or RTP.

Figure 3. Lineweaver-Burk (left) and a replot of [S]/Vo versus Vo (right) presentation of the inhibition of HIV RT. Enzyme activity was determined by measuring [^{32}P]dTTP incorporation in reaction mixtures (150 ul) as described for Fig. 1., except that the concentration of dTTP was varied. The reactions were incubated for 30 minutes at 37^{o}C, at which time 50 ul samples were removed for measurement. RTP concentration was zero (O) and 27 uM (O).

Figure 4. *In vitro* reverse transcriptase reactions in the presence of various combinations of AZT-TP and ribavirin compounds: 1, control with no drug and AZT-TP (16.6 uM); 2, RDP (49.5 uM) and combination of AZT-TP and RDP (the same concentration as in the reactions with only one drug); 3, RTP (62 uM) and combination of AZT-TP and RTP; 4, ribavirin (273 uM) and combination of AZT-TP and ribavirin. The conditions of the reaction mixtures (150 ul) were as for Fig. 1, and incubation was for 30 minutes at 37^{o}C at which time 50 ul were removed from each tube for measurement of acid insoluble radioactivity.

Table 1. Inhibition of AMV RT by ribavirin compounds. The reaction tubes each contained 150 ug/ml of the indicated drug and incubation was allowed to proceed for 30 minutes at 37^{O}C for 30 minutes, at which time 50 ul was removed from each tube for measurement of acid-insoluble radioctivity.

Drug	Percent [^{32}P]dTMP incorporation
None	100
RDP	0.3
RTP	18
Ribavirin	59